Some Aspects of Catalysis by the Amine Oxidase of Pea Seedlings*

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ABSTRACT: The affinity of purified pea seedling amine oxidase for oxygen was found to be dependent upon amine substrate concentration. Apparent K_m O_2 values for amine oxidase were 8.2×10^{-5} and 4.1×10^{-5} M at tryptamine concentrations of 1.0×10^{-3} and 2.0×10^{-4} M, respectively. A half-reaction for amine oxidase was demonstrated in the absence of O_2; approximately 1 mole of indoleacetaldehyde was formed anaerobically per mole of amine oxidase in the presence of tryptamine. An essential group at the active site of amine oxidase was titrated with essentially equimolar quantities of various hydrazines. The inhibition could not be reversed by dialysis. Hydrazines were shown to be irreversible competitive inhibitors; thus amine substrates could delay but not prevent amine oxidase inhibition. The data support the mechanism proposed earlier in which oxidative catalysis may involve the amine substrate reacting to form the corresponding aldehyde and a modified form of the enzyme which is subsequently oxidized by oxygen.

Amine oxidases appear to be either particulate enzymes with a flavin prosthetic group or soluble enzymes requiring Cu^{2+} and pyridoxal phosphate as prosthetic groups. However, PLP[1] has remained an elusive cofactor of the soluble amine oxidases (Fasella, 1967; Hill, 1967). Hill (1967) was unsuccessful in his attempts to demonstrate unequivocally the presence of PLP in AO.

The presence and requirement for Cu^{2+} in AO is well established (Mann, 1955, Hill and Mann, 1962, 1964) but its function is not understood. Cupric ions are required to activate copper-free amine oxidases from pea seedlings (Hill and Mann, 1964), bovine plasma (Yamada and Yasunobu, 1962), and pig kidney (Mondovi *et al.*, 1967). However, no change in valence state of copper has been observed by electron paramagnetic resonance spectra of these enzymes (Mondovi *et al.*, 1967, 1968; Goryachenkova *et al.*, 1968; Yamada *et al.*, 1963; Buffoni *et al.*, 1968) upon the anaerobic addition of amine substrates.

Purified pea-seedling AO is pink and absorbs maximally at 500 mμ (Mann, 1961; Hill and Mann, 1964). When substrate is added under anaerobic conditions, the color changes from pink to yellow and the absorption band at 500 mμ is replaced by bands with maxima at 466, 437.5, and 350 mμ. On oxygenation the yellow color changes to pink and the band at 500 mμ reappears at its original intensity. Mann and Hill have attributed the yellow color to the formation of an enzyme–substrate complex. Putrescine, ethylamine, lysine, and histamine all formed enzyme–substrate complexes which had identical absorption maxima at 466 mμ (Hill and Mann, 1964) even though the rates of formation of these complexes varied. Yellow complexes were not formed between these substrates and Cu^{2+}-free AO under anaerobic conditions. Hydrazine, when added to AO, replaced the 500-mμ absorption band with a band having a sharp maximum at 342 mμ (Hill and Mann, 1964).

The present study attempts to correlate the above spectral changes with possible reaction intermediates of AO. Time course patterns are presented which indicate that various hydrazines at approximately equimolar concentration compared to AO react rapidly and essentially irreversibly with AO. Amine substrates were found to delay but not prevent inhibition of AO, suggesting a reaction of the hydrazines with a functional group present at the catalytic site. AO was reacted with ^{14}C-labeled amine substrates under strict anaerobic conditions and approximately 1 mole of ^{14}C aldehyde was formed per mole of AO. It is proposed that AO catalysis involves a reaction of AO with amine substrates to form the corresponding aldehyde products and a modified form of the enzyme which is subsequently oxidized by molecular oxygen to regenerate the aldehyde form of AO. Certain apparent K_m values for AO substrates including O_2 have been determined. A preliminary report has appeared elsewhere (Reed and Swindell, 1969).

Materials and Methods

Pisum sativum var. Alaska seeds were purchased from Northrup, King & Co. Ammonium sulfate was a special enzyme grade obtained from Mann Research Laboratories. Ascorbic acid, sodium borohydride, hydrazine (anhydrous) 95+%, and 1,1-dimethylhydrazine (anhydrous) 99+% (UDMH) were obtained from Matheson Coleman & Bell. β-Hydroxyethylhydrazine (BOH) was obtained from Dr. S. I. Cohen of the Squibb Co. and from K & K Laboratories. Tryptamine hydrochloride, putrescine dihydrochloride, methylene blue, catalase (2 times crystallized from bovine liver), and pyridoxal phosphate were obtained from Sigma Chemical Co. Tryptophol was obtained from Regis Chemical Co. Indoleacetaldehyde as the sodium bisulfite addition product was obtained from Calbiochem. [2-^{14}C]Tryptamine

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[1] Abbreviations used are: AO, pea seedling amine oxidase; PLP, pyridoxal phosphate; BOH, β-hydroxyethylhydrazine; UDMH, 1,1-dimethylhydrazine; POPOP, 1,4-bis[2-(5-phenyloxazolyl)]benzene.

bisuccinate, varying in specific activity from 2.73 mCi/mmole to 10.2 mCi/mmole, [1,4-^{14}C]putrescine dihydrochloride, specific activity of 9.1 mCi/mmole and 5.22 mCi/mmole, and [1,5-^{14}C]cadaverine dihydrochloride, specific activity of 1.29 mCi/mmole, were obtained from New England Nuclear Corp. Tanks of compressed air and prepurified nitrogen were obtained from National Cylinder Gas Corp. of Chemetron Corp. Analyzed tanks of oxygen containing 1.07% O_2, and 1.08 ppm of O_2, and a balance of nitrogen were obtained from Matheson Co. POPOP and *p*-terphenyl of scintillation grade were obtained from Packard Instrument Co. Deionized glass-distilled water was used for all solutions. Purified pea seedling amine oxidase, with a specific activity of 48 units/mg (Hill and Mann, 1964), was the kind gift of Dr. P. J. G. Mann (Rothamsted Experimental Station, Harpenden, England). Specific activity of AO used for experiments described in Tables I, II, and III was 20 units/mg. Protein determinations were by a micro-Kjeldahl method (Lang, 1958).

Enzyme Assays. The method of Wurtman and Axelrod (1963) was used for the assay of AO activity with [2-^{14}C]tryptamine. In a typical reaction, 0.1 μCi (220,000 dpm) of [2-^{14}C]tryptamine, 40 mμmoles, 20 μg of catalase, and 0.1 ml of AO solution were in a total volume of 0.4 ml which was 33 mM each in potassium phosphate and sodium borate at pH 8 and contained in a 10-ml glass conical centrifuge tube. After a 10-min incubation at 25°, the reaction was terminated by the addition of 0.25 ml of 2 N HCl and extraction was accomplished with 5 ml of toluene by vigorous shaking, and centrifuging to separate the organic and aqueous phases. The organic phase (4 ml) was pipetted into 10 ml of fluor solution (30 mg of POPOP and 3 g of *p*-terphenyl per liter of toluene) and counted in a liquid scintillation counter (Packard Tri Carb 3375). Boiled enzyme was used for a control assay to determine the amount of radioactivity extracted by toluene.

The procedure of Okuyama and Kobayashi (1961) was used for the assay of AO with [1,4-^{14}C]putrescine. The procedure was the same as the Wurtman and Axelrod procedure except that the reaction was terminated by the addition of 100 mg of powdered sodium bicarbonate. The cyclized product, Δ^1-pyrroline, was extracted with toluene and counted as described for the tryptamine assay. Extraction of the product with toluene was completed within 2 min after sodium bicarbonate addition. This was necessary since a small amount of product (less than 15% of the total amount of product formed) was formed enzymically after sodium bicarbonate addition. In the anaerobic experiments, or those experiments in which the oxygen concentration was limited, the assay reaction mixture was boiled for 5 min prior to toluene extraction of aldehyde product.

Anaerobic and Varied O_2 Tension Atmospheres. Prepurified N_2 was passed through a 100-cm column of activated copper at 200° to remove oxygen according to the method of Meyer and Ronge (1939). The O_2-free N_2 was then saturated with water vapor prior to flushing the reaction vessel which was maintained at 1–2°. The N_2 stream flowed over the AO solution contained in a side arm of the reaction vessel and then bubbled through the ^{14}C amine solution and finally through a methylene blue and ascorbic acid solution for O_2 detection (Oster and Wotherspoon, 1954). The N_2 flow rate was measured with a rotameter (Manostat Corp. 36-541-03) and controlled by a needle valve. The entire apparatus was fabricated with a 0.25-in. diameter capillary tubing to permit assembly with nylon Swagelok fittings.

Sweeping the apparatus at a rate of 50–60 cc of N_2/min for 24–30 hr caused the O_2 concentration to be reduced ($<10^{-10}$ M) and the methylene blue solution to become colorless. The reaction between AO and ^{14}C amine substrate was then initiated by mixing after warming the reaction vessel to 25°. In a typical experiment, [2-^{14}C]tryptamine, 100 mμmoles (2.6×10^6 dpm), was treated with AO, 50 μg, approximately 0.5 mμmole based upon a molecular weight of 96,000 (Hill and Mann, 1964) and a purity of 90% (P. J. G. Mann, personal communication, 1967). The mixture was agitated with a small Teflon-coated magnet and after 10 or 20 min the reaction was terminated by boiling the reaction mixture prior to addition of acid and selective extraction of the [^{14}C]indoleacetaldehyde. Control experiments were conducted in the same manner but in the absence of AO.

When an O_2–N_2 mixture was desired for a particular oxygen tension rather than having an O_2-free N_2 system, an O_2 in N_2 gas mixture stream was flowed at a measured rate and then mixed with a metered O_2-free N_2 stream. The O_2 concentrations were calculated according to Henry's law (Jones, 1962).

Characterization of the Product of [2-^{14}C]Tryptamine Oxidation by AO. The reaction mixture consisted of AO, 61 μg, [2-^{14}C]tryptamine, 71.4 mμmoles containing 1.62×10^6 dpm, catalase, 140 μg, in a total volume of 2.8 ml of borate–phosphate buffer, pH 8.0, and incubated at 25° for 40 min. A 0.4-ml aliquot was transferred to a 10-ml centrifuge tube containing 5 mg of $NaBH_4$. After 30 min, 2.5 ml of 2 N HCl was added followed by 50 mg of twice-recrystallized tryptophol. After toluene extraction, the tryptophol was recrystallized from toluene–petroleum ether mixtures.

Results

Assay Methods. Aldehyde product formation as measured by ^{14}C assay was proportional to AO concentration when the incubation period was 60 min or less. When [1,4-^{14}C]putrescine (2.1×10^{-4} M, 251,000 dpm) was used as the substrate, the AO concentration was varied from 8 to 40 mμg in 0.4 ml of 33 mM borate–phosphate buffer, pH 8.0. With [2-^{14}C]tryptamine (1.0×10^{-4} M, 217,000 dpm) the AO concentration ranged from 0.2 to 4.0 μg. Catalase, 20 μg, was required to prevent marked inhibition of AO activity by the hydrogen peroxide formed in the assay mixture. Similar effects by hydrogen peroxide have been noted by Mann (1955) using a manometric assay procedure. Isotope dilution analysis after sodium borohydride reduction permitted indirect characterization of the toluene-extractable product from AO oxidation of [^{14}C]tryptamine as [^{14}C]indoleacetaldehyde. Sodium borohydride reduction was followed by extraction and addition of carrier tryptophol. Prior to recrystallization the disintegrations per minute per milligram of tryptophol was 3350. The disintegrations per minute per milligram with three consecutive crystallizations were 3140, 3080, and 3080, respectively.

Amine K_m Values. The apparent K_m value for tryptamine as a substrate for AO at pH 8 was found to be 4.0×10^{-4} M when the assay reaction mixture was in equilibrium with air (Figure 1). With this substrate, maximum AO activity was found to occur at pH 9.0 in borate–phosphate buffer. How-

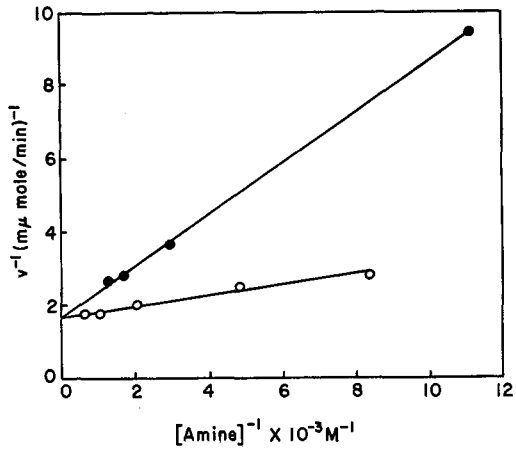

FIGURE 1: Lineweaver–Burk plots of the rates of tryptamine and putrescine oxidation by AO. [2-^{14}C]Tryptamine 217,000 dpm (●) with unlabeled tryptamine to give the desired substrate concentration was used to assay 4.0 μg of AO at pH 8.0. [1,4-^{14}C]Putrescine 251,000 dpm (○) with added unlabeled putrescine to give the desired concentrations was used to assay 0.040 μg of AO. Assay conditions were those described in the text.

ever, maximum enzyme stability was found to be at pH 7.8 to 8.0. The apparent K_m value for putrescine at pH 8.0 was found to be 7.4×10^{-5} M when the assay reaction mixture was equilibrated with air (Figure 1). Amine substrate concentrations ranging up to 10^{-3} M did not give any apparent substrate inhibition of AO activity.

Apparent K_m Values for Oxygen. The effect of dissolved oxygen concentration upon AO activity was found to be dependent upon amine substrate concentration (Figure 2). Apparent K_m O_2 values were 8.2×10^{-5} and 4.1×10^{-5} M at tryptamine concentrations of 1.0×10^{-3} and 2.0×10^{-4} M, respectively. The reciprocal plots obtained when the oxygen concentration was varied at the two fixed tryptamine concentrations show parallel lines for the initial rates of tryptamine

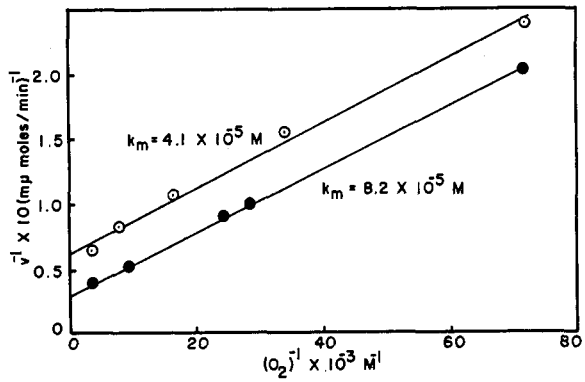

FIGURE 2: Lineweaver–Burk plots of the effect of oxygen concentration of the rate of tryptamine oxidation by AO. Oxygen tensions were established as described under Methods. [2-^{14}C]Tryptamine at 2.0×10^{-4} (⊙) and 1.0×10^{-3} M (●) concentrations and containing 131,000 and 262,000 dpm, respectively, was used to assay the activity of 50 μg of AO in a total volume of 1.1 ml of borate–phosphate buffer at pH 8.0. The incubations were for 10 min and the reaction stopped by placing the reaction vessel in boiling water for 5 min. Extraction of [2-^{14}C]indoleacetaldehyde for assay by liquid scintillation counting was as described under Methods.

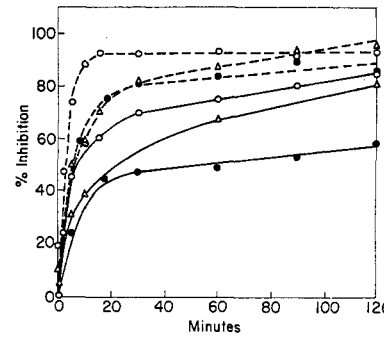

FIGURE 3: Time course plots of the inhibitory effects of incubation of AO with various hydrazines. [2-^{14}C]Tryptamine 233,000 dpm was added at the indicated time intervals to mixtures of 4.3 μg of AO, 20 μg of catalase, and hydrazines at concentrations of 1×10^{-7} M (-----) and 3.3×10^{-8} M (———). After 15 min at 25° the reaction mixtures were acidified and toluene was extracted in the usual manner for assay: hydrazine (●); β-hydroxyethylhydrazine (○); UDMH (△).

oxidation. The effect of N_2, if any, on the apparent K_m values for O_2 was not investigated.

Aldehyde formation from AO activity with O_2 concentrations of 10^{-7} to 10^{-9} M indicated that a small amount of aldehyde was being formed which could not be correlated with the level of O_2 tension. Therefore, aldehyde formation by AO activity in the strict absence of O_2 was examined for the possibility of a stoichiometric amount of aldehyde being formed in relation to the amount of AO present in the reaction mixtures.

Anaerobic Reaction of AO with Tryptamine. The reaction of AO, 0.5 mμmole, with ^{14}C-labeled tryptamine under anaerobic conditions ($<10^{-9}$ M O_2) resulted in the formation of approximately 1 mole of indoleacetaldehyde per mole of AO. Increasing the reaction time from 10 min to 20 min slightly decreased the recovery of indoleacetaldehyde: 0.46 ± 0.9 to 0.33 ± 0.05 mμmole. The decrease may possibly be due to nonspecific binding of the aldehyde to AO which prevented its extraction by toluene. The presence of catalase (20 μg) or increasing the O_2 concentration from 10^{-8} M to 10^{-9} M (10 ppm) did not change significantly the recovery of indoleacetaldehyde from the anaerobic reaction mixtures. Therefore, it must be concluded that in the absence of O_2 AO can react with the amine substrate to produce up to a mole of the corresponding aldehyde product per mole of AO. These results therefore indicate that AO has a carbonyl functional group which can react with the amino group of the amine substrate possibly *via* a reaction sequence similar to transamination to yield the aldehyde product and possibly an amino form of the enzyme. To further examine the role of such a carbonyl functional group in AO catalysis, stoichiometric reactions between AO and hydrazines were examined in a series of mutual depletion kinetic studies.

Time Course of AO Inhibition by Hydrazines. The reaction of hydrazines with AO in the absence of amine substrate was studied as a function of time (Figure 3). The molar quantity of each of the hydrazines employed was approximately the same as that of AO present in each reaction mixture. The reactions were quite rapid and when hydrazines were present in a substoichiometric concentration, the total reaction time required for maximum inhibition was approximately 2 hr.

TABLE I: Effect of Dialysis upon the AO Inhibition by Hydrazines.[a]

Inhibitor	Concn (M)	Per Cent Inhibition: Before Dialysis	Per Cent Inhibition: After Dialysis
BOH	1.0×10^{-7}	99	96
	3.3×10^{-8}	83	78
UDMH	1.0×10^{-7}	96	97
	3.3×10^{-8}	67	68
Hydrazine	1.0×10^{-7}	91	88
	3.3×10^{-8}	40	26

[a] AO, 19 μg, and inhibitor in a total volume of 0.9 ml of 33 mM borate–phosphate buffer, pH 8.0. After incubation at 25° for 2 hr, a 0.2-ml aliquot was assayed and the remaining reaction mixture dialyzed against 100 ml of buffer at pH 8.0 for 18 hr with one change in buffer. Control reaction mixtures contained buffer in lieu of hydrazines.

However, a major portion of the inhibition occurred within the initial 20–30 min of the reaction. While all of the hydrazines examined were found to have a very high affinity for AO, β-hydroxyethylhydrazine had the greatest affinity, hydrazine next, and finally UDMH with a somewhat lesser affinity. With the assumption of approximately equimolar concentrations of hydrazine and AO, second-order rate constants have been estimated. Plots of the reciprocal of per cent remaining activity *vs.* time in minutes gave rate constants which were 2×10^4 M^{-1} sec^{-1} for hydrazine and UDMH and a value of 1×10^6 M^{-1} sec^{-1} for BOH.

Dialysis of AO–Hydrazine Reaction Mixtures. The reaction of AO with hydrazines in the absence of substrate appeared to be essentially irreversible as evidenced by the stoichiometry exhibited. Therefore, it was decided to examine whether or not the inhibition of AO by hydrazines could be reversed by dialysis. AO was treated for a 2-hr period with concentrations of hydrazines which in one set of experiments gave complete inhibition of AO and in a second set of experiments only partial inhibition of AO. The reaction mixtures were then dialyzed extensively. Assay of AO activity before and after dialysis (Table I) gave evidence that the inhibition was essentially nonreversible by dialysis. The partial inhibition which was observed with the hydrazines at 3.3×10^{-8} M concentration again indicates that the three hydrazines are approximately equal in their ability to inhibit AO. From these data one must conclude that a substoichiometric amount of any of these three hydrazines in the presence of AO would result in the formation of enzyme–inhibitor complexes, with no free hydrazines being present in the reaction mixture after approximately 1–2 hr of reaction time. AO did not change in activity after dialysis for 24 hr (Table II). The prosthetic groups are tightly bound (Mann, 1961) and therefore AO was not stimulated by the addition of either Cu^{2+} or pyridoxal phosphate. As was shown by Hill and Mann (1962, 1964) Cu^{2+} was a fairly potent inhibitor of AO; a 10^{-5} M concentration caused about 75% inhibition of AO activity.

Reactions of Hydrazines and AO in the Presence of Amine Substrate. When AO was added to a mixture of tryptamine and any of the three hydrazines, it was observed that the substrate appeared to protect the enzyme; much higher concentrations of hydrazines were necessary to cause the same degree of inhibition as observed in those experiments in which the hydrazine was treated with AO prior to addition of substrate (Table III). Approximately 100-fold greater concentrations of the hydrazine were necessary to cause an immediate and complete inhibition of AO activity when compared with the concentrations of hydrazines necessary to inhibit AO after a 2-hr reaction period (Tables II and III). These and other experiments have led to the conclusion that

TABLE II: Effects of Cu^{2+}, Pyridoxal Phosphate, and Dialysis on AO Activity.[a]

Addition	Concn (M)	Indoleacetaldehyde Formed (mμmoles): Undialyzed AO	Indoleacetaldehyde Formed (mμmoles): Dialyzed AO (24 hr)
None		4.3	4.0
Pyridoxal phosphate	10^{-4}	3.0	3.7
Pyridoxal phosphate	10^{-5}	4.1	3.9
Cu^{2+}	10^{-4}	0.5	
Cu^{2+}	10^{-5}	1.0	0.8
Cu^{2+}	10^{-6}		3.2
Pyridoxal phosphate and Cu^{2+}	10^{-5}	0.9	

[a] AO, 4.3 μg, was incubated for 10 min at 25° with [2-^{14}C]-tryptamine (233,000 dpm), 50 mμmoles, with the additions noted in a final reaction volume of 0.4 ml of 0.033 M borate–phosphate buffer, pH 8.0. Dialysis was in 100 ml of 0.033 M borate–phosphate buffer, pH 8.0, for 17 hr at 1 to 2°.

TABLE III: Inhibition of AO Activity When AO Is Added to a Mixture of Substrate and Inhibitor.[a]

Inhibitor Concn (M)	Per Cent Inhibition: Hydrazine	Per Cent Inhibition: BOH	Per Cent Inhibition: UDMH
10^{-5}	98	98	80
10^{-6}	74	90	48
10^{-7}	18	43	
3.3×10^{-8}	6	38	23
10^{-8}	4	10	24

[a] AO, 4.3 μg, was added to a mixture of [2-^{14}C]tryptamine, 1.26×10^{-4} M, containing 232,800 dpm of ^{14}C inhibitor, 20 μg of catalase, and 0.033 M borate–phosphate buffer, pH 8.0, to give a final volume of 0.4 ml. Assay mixtures were incubated at 25° for 10 min.

the amine substrate and hydrazine compete for the same active site on AO.

Discussion

The results in this paper are in accordance with a mechanism in which a modified form of AO is produced. Reciprocal plots in which the slopes are unchanged regardless of the concentration of the second substrate are consistent with a mechanism of oxidative deamination in which the reaction proceeds through a modified form of the enzyme and a series of complexes without the formation of kinetically significant amounts of a ternary complex (Tipton, 1968). Such a mechanism has been termed a ping-pong mechanism by Cleland (1963). Anaerobic formation of approximately stoichiometric quantities of indoleacetaldehyde by AO from tryptamine indicates the formation of a modified enzyme which results from an initial enzyme–substrate complex, in agreement with the mechanistic scheme presented by McEwen *et al.* (1966) for rabbit plasma amine oxidase. Finazzi-Agro *et al.* (1969) have reported evidence for the anaerobic release of 0.9 mole of aldehyde per mole of hog kidney diamine oxidase in the presence of cadaverine and they suggest a ping-pong mechanism for this enzyme. The nature of the carbonyl group of AO remains in question (Hill, 1967) but the irreversible titration of AO with near equimolar quantities of various hydrazines leaves little doubt concerning the role it has in the initial reaction with amine substrates. Similar results have been found for bovine plasma amine oxidase (Reed and Swindell, 1969).

Competitive inhibition experiments indicate that amine substrates can delay but not prevent AO inhibition by hydrazines. Hill and Mann (1964) described the changes in the absorption spectrum of AO produced by hydrazines; addition of hydrazine changed the color of the enzyme from pink to yellow. The initial change was the replacement of the 500-mμ band with a sharp maximum at 333 mμ and a much less intense band with a maximum at 415–430 mμ. On standing and exposure to air at room temperature, the solution slowly lost its yellow color and after 90 min the 415–430-mμ band had disappeared and the maximum of the other band had shifted to 342 mμ and intensified. This band reached a maximum in 5–6 hr and then slowly decreased. This evidence along with the titration experiments reported here indicate that the amine substrate does interact with a carbonyl functional group on AO which is very sensitive to hydrazines and which appears to form enzyme–inhibitor complexes which cause irreversible inhibition. Whether the inhibition is oxygen dependent is not known.

The role of copper remains unclear. The formation of a proposed modified form of AO would suggest that oxygen binds in some manner to the modified form of the enzyme to receive two electrons to form H_2O_2. Addition of water to the oxidized AO intermediate would yield NH_3 and regenerated enzyme. Such a mechanism would be in agreement with the observed lack of valence change of copper in several amine oxidases (Yamada and Yasunobu, 1962; Mondovi *et al.*, 1967, 1968; Goryachenkova *et al.*, 1968; Buffoni *et al.*, 1968). Mondovi *et al.* (1969) have reported a 60% reduction of the intensity of the copper signal 170 sec after anaerobic mixing of cadaverine with pig kidney diamine oxidase. Whether this slow valence change of copper is part of the catalytic mechanism is yet to be determined. It may represent a reaction of a modified form of the enzyme rather than one of an enzyme–substrate complex.

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